IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Patent Application of

KOEBERL et al — Atty. Ref.: 01579-1155

Serial No. 10/761,530 — TC/A.U.: 1652

Filed: January 21, 2004 — Examiner: Raghu, G.

For: CONSTRUCTS FOR EXPRESSING LYSOSOMAL POLYPEPTIDES

* *

June 14, 2010

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Appellants hereby appeal the final rejection of claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82, in the Office Action dated August 13, 2009, and submit the present Appeal Brief pursuant to 37 CFR § 41.37. A Notice of Appeal was filed January 13, 2010, the period for filing an Appeal Brief having been extended up to June 13, 2010 by submission of the required petition and fee herewith.

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(i) REAL PARTY IN INTEREST

The real party in interest is Duke University, Durham, North Carolina 27708-0083, by way of an Assignment from the inventors to Duke University, Durham, North Carolina 27708-0083, recorded in the U.S. Patent and Trademark Office on April 19, 2004, at Reel 015224, Frame 0557.

(II) RELATED APPEALS AND INTERFERENCES

Appellants, Appellants' legal representative, and the assignee are not aware of any related prior or pending appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(III) STATUS OF CLAIMS

Claims 1-5, 8-12, 14-16, 18, 21, 22, 24-73, 75-77 and 79-82 are pending. Claims 6, 19 and 20 were cancelled in the Amendment filed March 15, 2007, and claims 7, 13, 17, 23, 74 and 78 were cancelled in the Amendment filed September 24, 2007. Claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82 have been finally rejected. Claims 30-72 stand withdrawn from consideration.

Claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82 are the subject of the present appeal. A copy of claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82 is attached as a Claims Appendix.

It is noted that claim 22 stands rejected in the Office Action dated August 13, 2009, however, on page 1 of that Action, it is not included in the listing of rejected claims.

(IV) STATUS OF AMENDMENTS

No Amendment Under Rule 116 has been filed in response to the final Office Action dated August 13, 2009.

(V) SUMMARY OF CLAIMED SUBJECT MATTER

The present invention, as claimed in claim 1 (from which claims 2-5, 8-12, 14-16, 18, 21, 22, 24-29, 77 and 79 depend) relates to an isolated nucleic acid encoding a chimeric polypeptide. The chimeric polypeptide comprises a secretory signal sequence operably linked to human acid α-glucosidase (GAA). In accordance with the invention of claim 1, the secretory signal sequence replaces the leader sequence of native human GAA.

Support for the invention of claim 1, is found throughout the application, including, for example, in claims 1 and 7 as originally filed, at page 23, lines 1-3 and in Example 13.

The present invention, as claimed in claim 73 (from which claims 75, 76 and 80-82 depend), relates to an isolated nucleic acid encoding a chimeric polypeptide comprising a secretory signal sequence operably linked to human GAA. The secretory signal sequence comprises a sequence selected from the group consisting of the sequences of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:9 and the secretory signal sequence replaces the leader sequence of native human GAA.

Support for the invention of claim 73 is found, for example, in claims 1, 3 and 7 as originally filed, at page 23, lines 1-3 and in Example 13.

(VI) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are presented for review:

Whether claims 1, 2, 8-11, 18, 21, 22 and 24-29 are anticipated under 35 USC 102(b) by Van Bree et al (WO 00/34451).

Whether claims 5, 12, 14-16, 73, 75 and 80-82 would have been obvious under 35 USC 103(a) over Van Bree et al in view of Amalfitano et al (WO 02/098466).

Whether claims 3, 4, 73, 75, 77 and 79 would have been obvious under 35 USC 103(a) over Van Bree et al, in view of Amalfitano et al and further in view of Heus (USP 6,858,425) and Haseltine (WO 2005/003296).

Whether claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75, 77 and 79-82 would have been obvious under 35 USC 103(a) over Van Bree et al, Amalfitano et al, Heus, Haseltine and further in view of Martin et al (WO 00/47741).

Whether claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82 would have been obvious under 35 USC 103(a) over Van Bree et al, Amalfitano et al, Heus, Haseltine et al, Martin et al and further in view of Whitfeld et al (USP 5,298,400).

Whether claims 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 and 79-82 would have been obvious under 35 USC 103(a) over Van Bree et al, Amalfitano et al, Heus, Haseltine et al, Martin et al, Whitfeld et al and further in view of Meulien (USP 5,521,070).

(VII) ARGUMENT

REJECTION OF CLAIMS 1, 2, 8-11, 18, 21, 22 and 24-29 UNDER 35 USC 102(b) AS ANTICIPATED BY VAN BREE ET AL

Reversal of the rejection of the claims as anticipated is requested for the reasons that follow.

Claim 1 (from which the other claims subject to this rejection depend) relates to a nucleic acid encoding a chimeric polypeptide comprising a secretory signal sequence operably linked to human GAA. Claim 1 requires that the secretory signal sequence replace the leader sequence of native human GAA. It is submitted that Van Bree et al includes no such teaching.

A rejection under 35 USC 102 is proper only when the subject matter of the claims is identically disclosed/described in the cited art.

> Thus, it is not enough that the prior art reference discloses part of the claimed invention, which an ordinary artisan might supplement to make the whole, or that it includes multiple, distinct teachings that the artisan might somehow combine to achieve the claimed invention. See Arkley, 455 F.2d at 587 ("[T]he [prior art] reference must clearly and unequivocally disclose the claimed [invention] or direct those skilled in the art to the [invention] without any need for picking, choosing, and combining various disclosures not directly related to each other by the teachings of the cited reference.").
>
> Net MoneyIn, Inc. v. VeriSign, Inc., 545 F.3d 1359, 1371 (Fed Cir. 2008)

Appellants respectfully submit that Van Bree et al does not "clearly and unequivocally" disclose the claimed invention (or direct an artisan to it) "without any

need for picking, choosing and combining various disclosures not directly related to each other by the teachings of the cited reference".

On the contrary, the Examiner relies on general teachings in Van Bree et al relating to lysosomal protein processing (page 7, lines 26-32), teachings relating to selection of a signal sequence capable of directing secretion of a lysosomal protein (page 9, lines 16-30), and a teaching of the replacement of the secretion signal sequence linked to the lysosomal protein coding sequence with a signal sequence that targets the processing enzyme to the endoplasmic reticulum without secretion (page 11, lines 29-35). The Examples provided in Van Bree et al make reference to the human acid α glucosidase gene. However, no reference is found in those Examples (or elsewhere in Van Bree et al) to a nucleic acid encoding a chimeric polypeptide comprising a secretory signal sequence operably linked to human acid α-glucosidase (GAA) wherein that secretory signal sequence replaces the leader sequence of the native human GAA, as required by the claims. The statement on page 5, lines 26-29, of Van Bree et al to which the Examiner referes does not cure this deficiency.

As the citation does not "clearly and unequivocally" disclose the invention, reversal of the rejection is clearly in order and same is requested.

REJECTION OF CLAIMS 5, 12, 14-16, 73, 75 AND 80-82 UNDER 35 USC 103 AS OBVIOUS OVER VAN BREE ET AL IN VIEW OF AMALFITANO ET AL

Reversal of the rejection of the claims as obvious is requested for the reasons that follow.

The fundamental failings of Van Bree et al are discussed above, those comments being incorporated here by reference. Amalfitano et al also fails to teach the required replacement of the leader sequence of human GAA with a secretory signal sequence.

In rejecting the claims as obvious, the Examiner makes reference to the paragraph beginning at line 13 on page 22 of Amalfitano et al. The referenced portion of the citation indicates that an adenovirus vector can be used to infect a cell in culture to produce a polypeptide of interest, lysosomal acid α-glucosidase is given as an example (at page 28, to which the Examiner also refers, reference is made to human GAA). While the Examiner cites pages 3-41 of Amalfitano et al generally and, especially, pages 6, 7, 12, 22, 26, 28-30, 35 and 41, and Examples 1, 4, 9 and 13, the Examiner fails to indicate where Amalfitano et al teaches or would have suggested replacing the leader sequence of native human GAA with a secretory signal sequence, as required by the claims.

In view of the fundamental deficiencies of Van Bree et al and Amalfitano et al, their combination would not have brought one skilled in the art any closer to the claimed invention. Accordingly, reversal of the rejection is requested.

REJECTION OF CLAIMS 3, 4, 73, 75, 77 AND 79 UNDER 35 USC 103 AS OBVIOUS OVER VAN BREE ET AL, AMALFITANO ET AL IN VIEW OF HEUS AND HASELTINE ET AL

Reversal of the rejection of the claims as obvious is requested for the reasons that follow.

The deficiencies of Van Bree et al and Amalfitano et al are discussed above, those comments being incorporated here by reference.

The present invention results, at least in part, from studies designed to test the hypothesis that chimeric lysosomal polypeptides containing an alternative signal peptide could increase the secretion of lysosomal polypeptides from transduced cells and enhance receptor-mediated uptake of lysosomal polypeptides in tissues. The data presented in the application (and in Sun et al, Mol. Ther. 14:822 (2006) (of record) – a copy of which is submitted herewith) make it clear that replacement of the lysosomal leader sequence (which targets the polypeptide to the lysosome) by a secretory signal peptide increased secretion from cultured cells (see, for example, Fig. 15 of the application). Furthermore, receptor mediated uptake of the chimeric polypeptide occurred efficiently (see, for example, Table 1 of Sun et al, Mol. Ther. 14:822 (2006)). The uptake was inhibited by mannose-6-phosphate thereby implicating the involvement of mannose-6-phosphate receptors.

As discussed above, neither Van Bree et al nor Amalfitano et al teach or would have suggested replacement of the leader sequence of native human GAA with a secretory signal sequence. Nothing in the teachings of Heus relating to 3' untranslated

region sequences of GAA or in Haseltine et al's teaching of the albumin signal sequence provide such a teaching or suggestion.

Appellants submit that it is only with hindsight that the cited references would have been combined - the documents themselves would not have suggested their combination. Accordingly, the rejection is clearly not well founded and reversal of same is requested.

REJECTION OF CLAIMS 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75, 77 AND 79-82 UNDER 35 USC 103 AS OBVIOUS OVER VAN BREE ET AL, AMALFITANO ET AL, HEUS, HASELTINE ET AL AND FURTHER IN VIEW OF MARTIN ET AL

Reversal of the rejection of the claims as obvious is requested for the reasons that follow.

The deficiencies of Van Bree et al and Amalfitano et al are discussed above, those comments being incorporated herein by reference. Nothing in the teachings of Heus relating to 3' untranslated region sequences of GAA, in Haseltine et al's teaching of the albumin signal sequence or in Martin et al's teaching of an erythropoietin signal peptide would have cured the fundamental failings of Van Bree et al and Amalfitano et al. In addition, the documents cited here, like those cited above, would only have been combined by one having benefit of the present invention.

Reversal of the rejection is clearly in order and the same is requested.

REJECTION OF CLAIMS 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 AND 79-82 UNDER 35 USC 103 AS OBVIOUS OVER VAN BREE ET AL, AMALFITANO ET AL, HEUS, HASELTINE ET AL, MARTIN ET AL AND FURTHER IN VIEW OF WHITFELD ET AL

Reversal of the rejection of the claims as obvious is requested for the reasons that follow.

The failings of Van Bree et al and Amalfitano et al are detailed above, those comments being incorporated by reference. Nothing in the teachings of Heus relating to 3' untranslated region sequences of GAA, in Haseltine et al's teaching of the albumin signal sequence or in Martin et al's teaching of an erythropoietin signal peptide would have cured those failings, likewise, nothing in Whitfeld et al's teachings relating to a therapeutic polypeptide comprising an α-1-antitrypsin secretory signal sequence. Reversal of the rejection is, therefore, requested.

REJECTION OF CLAIMS 1-5, 8-12, 14-16, 18, 21, 22, 24-29, 73, 75-77 AND 79-82 UNDER 35 USC 103 AS OBVIOUS OVER VAN BREE ET AL, AMALFITANO ET AL, HEUS, HASELTINE ET AL, MARTIN ET AL, WHITFELD ET AL AND FURTHER IN VIEW OF MEULIEN

Reversal of the rejection is requested for the reasons that follow.

The deficiencies of Van Bree et al and Amalfitano et al are described above, those comments being incorporated here by reference. It is also pointed out above that nothing in Heus, Haseltine et al, Martin et al or Whitfeld et al would have suggested replacing of the leader sequence of native human GAA with a secretory signal sequence. Indeed, the Examiner does not contend otherwise. Meulien's teaching of a Factor IX secretory signal sequence certainly would not have provided such a suggestion.

Appellants submit that the documents cited here, like those cited above, would only have been combined by one having benefit of the present invention. Reversal of the rejection is, therefore, requested.

CONCLUSION

In conclusion it is believed that the application is in clear condition for allowance; therefore, early reversal of the Final Rejection and passage of the subject application to issue are earnestly solicited.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: /Mary J. Wilson/
Mary J. Wilson
Reg. No. 32,955

MJW:tat
901 North Glebe Road, 11th Floor
Arlington, VA 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

(VIII) CLAIMS APPENDIX

1. An isolated nucleic acid encoding a chimeric polypeptide comprising a secretory signal sequence operably linked to human acid α-glucosidase (GAA), wherein said secretory signal sequence replaces the leader sequence of native human GAA.

2. The isolated nucleic acid of Claim 1, wherein said secretory signal sequence is a secretory signal sequence of a secreted polypeptide.

3. The isolated nucleic acid of Claim 2, wherein said secretory signal sequence is an erythropoietin, Factor IX, albumin or α1-antitrypsin precursor polypeptide secretory signal sequence.

4. The isolated nucleic acid of Claim 1, wherein said secretory signal sequence comprises the amino acid sequence of SEQ ID NO:5.

5. The isolated nucleic acid of Claim 1, wherein said isolated nucleic acid is operatively linked to a transcriptional control element operable in liver cells.

8. (Previously Presented) The isolated nucleic acid of Claim 1, wherein said isolated nucleic acid further comprises a 3' untranslated region.

9. The isolated nucleic acid of Claim 8, wherein:

(a) said 3' untranslated region comprises a deletion therein; or

(b) wherein said 3' untranslated region is less than 200 nucleotides in length and comprises a segment that is heterologous to said GAA coding region.

10. The isolated nucleic acid of Claim 1, wherein the isolated nucleic acid is 4 kilobases or less in length.

11. A vector comprising the isolated nucleic acid of Claim 1.

12. The vector of Claim 11, wherein said vector is an adeno-associated virus (AAV) vector.

14. A pharmaceutical formulation comprising the isolated nucleic acid of Claim 1 in a pharmaceutically acceptable carrier.

15. The pharmaceutical formulation of Claim 14, wherein said pharmaceutical formulation comprises a vector comprising the isolated nucleic acid.

16. The pharmaceutical formulation of Claim 15, wherein said vector is an adeno-associated virus (AAV) vector.

18. A cell comprising the isolated nucleic acid of Claim 1.

21. A method of delivering a nucleic acid encoding human GAA to a cell, comprising contacting a cell with the isolated nucleic acid according to Claim 1 under conditions sufficient for the isolated nucleic acid to be introduced into the cell and expressed so that the chimeric polypeptide comprising the secretory signal sequence operably linked to human GAA is produced, and the human GAA is secreted from the cell.

22. The method of Claim 21, wherein the cell is contacted with a vector comprising the isolated nucleic acid.

24. The method of Claim 21, wherein the cell is a cultured cell.

25. The method of Claim 21, wherein the cell is a cell *in vivo*.

26. A method of producing human GAA in a cultured cell, comprising:

contacting a cultured cell with the isolated nucleic acid according to Claim 1 under conditions sufficient for the isolated nucleic acid to be introduced into the cultured cell and expressed so that the chimeric polypeptide comprising the secretory signal sequence operably linked to human GAA is produced, and the human GAA is secreted from the cultured cell into a cell culture medium, and

collecting the human GAA secreted into the cell culture medium.

27. The method of Claim 26, wherein the cell is a mammalian cell.

28. The method of Claim 27, wherein the cell is a CHO cell, a 293 cell, a HT1080 cell, a HeLa cell or a C10 cell.

29. The method of Claim 26, wherein the cell is contacted with a vector comprising the isolated nucleic acid.

73. An isolated nucleic acid encoding a chimeric polypeptide comprising a secretory signal sequence operably linked to human GAA, wherein the secretory signal sequence comprises a sequence selected from the group consisting of the sequences of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:9, and wherein said secretory signal sequence replaces the leader sequence of native human GAA.

75. The isolated nucleic acid according to claim 73 wherein said human GAA has an amino acid sequence corresponding to residues 28-952 of SEQ ID NO:2.

76. The isolated nucleic acid according to claim 73 wherein said secretory signal sequence comprises the sequence of SEQ ID NO:8 or SEQ ID NO:9.

77. The method according to claim 26 wherein said secretory signal sequence comprises a sequence selected from the group consisting of the sequences of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:9.

79. The isolated nucleic acid of claim 8, wherein said 3' untranslated region comprises nucleotides 2878 to 3012 of SEQ ID NO:3.

80. A vector comprising the isolated nucleic acid according to claim 75.

81. A pharmaceutical formulation comprising the isolated nucleic acid according to claim 75 and a carrier.

82 The pharmaceutical formulation according to claim 81, wherein said pharmaceutical formulation comprises a vector comprising the isolated nucleic acid.

(IX) EVIDENCE APPENDIX

Sun et al, "Enhanced Efficacy of an AAV Vector Encoding Chimeric, Highly Secreted Acid α-Glucosidase in Glycogen Storage Disease Type II", Mol. Ther. 14:822 (2006)

(X) RELATED PROCEEDINGS APPENDIX

(NONE)

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Enhanced Efficacy of an AAV Vector Encoding Chimeric, Highly Secreted Acid α-Glucosidase in Glycogen Storage Disease Type II

Baodong Sun,[1] Haoyue Zhang,[1] Daniel K. Benjamin Jr.,[2] Talmage Brown,[3] Andrew Bird,[1] Sarah P. Young,[1] Alison McVie-Wylie,[4] Y.-T. Chen,[1] and Dwight D. Koeberl[1,*]

[1]*Division of Medical Genetics, Department of Pediatrics and* [2]*Department of Pediatrics and Duke Clinical Research Institute, Duke University Medical Center, Durham, NC 27710, USA*
[3]*College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27695, USA*
[4]*Genzyme Corporation, Framingham, MA 01701, USA*

To whom correspondence and reprint requests should be addressed at DUMC Box 3528, Bell Building, Duke University Medical Center, Durham, NC 27710, USA. Fax: +1 919 684 2362. E-mail: dwight.koeberl@duke.edu.

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Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) is an inherited muscular dystrophy caused by deficiency in the activity of the lysosomal enzyme acid α-glucosidase (GAA). We hypothesized that chimeric GAA containing an alternative signal peptide could increase the secretion of GAA from transduced cells and enhance the receptor-mediated uptake of GAA in striated muscle. The relative secretion of chimeric GAA from transfected 293 cells increased up to 26-fold. Receptor-mediated uptake of secreted, chimeric GAA corrected cultured GSD-II patient cells. High-level hGAA was sustained in the plasma of GSD-II mice for 24 weeks following administration of an AAV2/8 vector encoding chimeric GAA; furthermore, GAA activity was increased and glycogen content was significantly reduced in striated muscle and in the brain. Administration of only 1×10^{10} vector particles increased GAA activity in the heart and diaphragm for >18 weeks, whereas 3×10^{10} vector particles increased GAA activity and reduced glycogen content in the heart, diaphragm, and quadriceps. Furthermore, an AAV2/2 vector encoding chimeric GAA produced secreted hGAA for >12 weeks in the majority of treated GSD-II mice. Thus, chimeric, highly secreted GAA enhanced the efficacy of AAV vector-mediated gene therapy in GSD-II mice.

Key Words: Pompe disease, Gene therapy, adeno-associated virus vector, signal peptide, acid α-glucosidase, glycogen storage disease

Introduction

Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20). Massive accumulation of glycogen in lysosomes of striated muscle disrupts cellular functions. Clinically, the disease manifestations include muscle weakness, hypotonia, hypertrophic cardiomyopathy, and respiratory failure [1].

GAA is an exo-1,4-α-D-glucosidase that hydrolyzes both α-1,4 and α-1,6 linkages of oligosaccharides to liberate glucose during the complete degradation of glycogen in lysosomes. Human GAA (hGAA) is synthesized as a 110-kDa precursor form, which matures through an approximately 90-kDa endosomal intermediate into the final lysosomal 76- and 67-kDa forms [2–5]. GAA is normally absent from plasma by Western blot analysis [6].

Proteins are routed specifically to secretory or endocytic pathways during synthesis in the endoplasmic reticulum and traverse the Golgi network [7]. The signal peptide of a secreted protein is recognized during translation by the signal recognition peptide; subsequently, the signal peptide is cleaved by a membrane-bound signal peptidase, and the mature secreted protein enters a secretory vesicle [8]. Secretory vesicles are coated by COPII, possibly generated in response to the nascent secretory protein (cargo) [9,10]. Thus, secreted proteins are destined for the secretory pathway by recognition of their signal peptides, whereas lysosomal proteins are routed differently through the endocytic pathway to arrive in lysosomes [7,8].

Intravenous administration of adenovirus vectors encoding GAA previously demonstrated generalized correction of glycogen storage in the GAA knockout (GAA-KO) mouse model for Pompe disease [11,12], although

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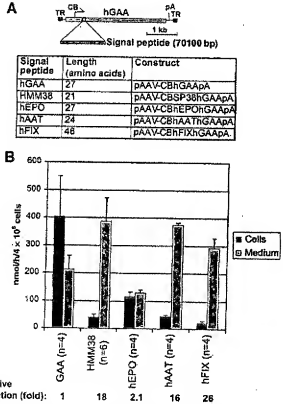
glycogen gradually reaccumulated in the months following vector administration [13]. A transgene containing a universal (CB) promoter demonstrated the secretion of hGAA when delivered within AAV2/2, AAV2/6, and AAV2/8 vectors in immunodeficient, GAA-KO/SCID mice; however, this was not feasible in immunocompetent GAA-KO mice due to anti-hGAA antibody production [6,14]. Even when GAA-KO mice were tolerized to hGAA by neonatal administration of the recombinant enzyme, a subset of those mice still formed anti-GAA antibodies in response to administration of an AAV2/8 vector encoding hGAA [15]. The appearance of anti-GAA antibodies correlated with the disappearance of secreted hGAA precursor from the plasma and a lack of efficacy from viral vectors [13,16].

Secreted proteins are directed extracellularly by the presence of an N-terminal signal peptide [17]. One strategy to increase the secretion of lysosomal enzymes is modifying the signal peptide. Other methods of modifying proteins to increase secretion include insertion of alternative sequences such the HIV Tat protein that could modify the structure and/or function of the protein, thereby compromising the protein's activity and therapeutic effect [18,19]. We have investigated the hypothesis that a chimeric lysosomal enzyme containing an alternative signal peptide will achieve much higher secretion of the recombinant lysosomal enzyme from transduced cells in GAA-KO mice.



Signal peptide	Length (amino acids)	Construct
hGAA	27	pAAV-CBhGAApA
HMM38	21	pAAV-CBSP38hGAApA
hEPO	27	pAAV-CBhEPOhGAApA
hAAT	24	pAAV-CBhAAThGAApA
hFIX	46	pAAV-CBhFIXhGAApA

FIG. 1. Chimeric hGAA production in cells and medium. (A) Vector plasmids encoding chimeric hGAA. The native signal peptide was replaced by the indicated signal peptides in the hGAA cDNA. (B) 293 cells were transfected with AAV vector plasmids containing chimeric hGAA cDNAs and were collected 40 h posttransfection. Total hGAA activity was assayed in the cells and in the medium. Mean and standard deviation for the indicated number of replicate experiments are shown. Relative secretion was calculated as the proportion of measured hGAA secreted (medium/cells) divided by the proportion secreted by the vector plasmid containing native hGAA.

Results and Discussion

We screened several signal peptides for the ability to increase higher level hGAA secretion from cultured human cells. We replaced the signal peptide for hGAA by other signal peptides in the hGAA cDNA contained in an adeno-associated virus (AAV) vector plasmid, including a synthetic signal peptide (HMM38 [17]), human erythropoietin (hEPO), human prealbumin (hAlb), human α1-antitrypsin (hAAT), or human coagulation factor IX (hFIX) (Fig. 1A). We analyzed the secretion of chimeric hGAA in transiently transfected 293 cells (Fig. 1B). The ratio of secreted hGAA (in the medium) to nonsecreted hGAA (in the cells) increased markedly for the chimeric hGAAs. The HMM38, hFIX, and hAAT signal peptides increased the relative secretion of chimeric hGAA between 16- and 26-fold compared to the native hGAA. We chose the vector plasmid containing the hAAT signal peptide for further evaluation, because levels of secreted hGAA were as high or higher than those for the other chimeric hGAA cDNAs (Fig. 1B).

We analyzed the receptor-mediated uptake of chimeric hGAA in cultured fibroblasts from a Pompe disease patient. The chimeric hGAA was taken up efficiently by patient fibroblasts, and the uptake was inhibited by approximately 80% in the presence of mannose 6-phosphate (Table 1). The intracellular glycogen content was significantly reduced by the addition of chimeric hGAA in the medium, compared to untreated control cells; moreover, this decrease in glycogen was blocked by mannose 6-phosphate (Table 1). Mannose 6-phosphate receptors were implicated in the uptake of chimeric hGAA, because mannose 6-phosphate blocked the biochemical correction of patient cells just as it did for native hGAA [20].

Secreted, 110-kDa precursor hGAA was clearly elevated by Western blot analysis for the 293 cells transfected with the chimeric hGAA expression vectors (Fig. 2A, lanes 5–12), compared to 293 cells containing the native hGAA expression vector (Fig. 2A, lanes 3 and 4). The vector containing the hEPO signal peptide increased GAA activity in the medium only twofold (Fig. 1A), because the majority of that chimeric hGAA was retained intracellularly (Fig. 2A). Secreted 110-kDa hGAA precursor was more reliably detected by Western blotting than by the GAA enzyme assay, because the specific activity of

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TABLE 1: Enzyme uptake in GSD-II patient fibroblasts[a]

Treatment	GAA activity (range) (nmol/h/mg)	Glycogen content (range) (μmol glucose/mg protein)
Untreated	1.50 (1.09–1.87)	0.55 (0.54–0.56)
SP-hGAA	41.47 (37.73–47.18)	0.33 (0.32–0.34)
SP-hGAA+mannose 6-phosphate	8.27 (6.12–12.04)	0.56 (0.54–0.58)

[a] Cultured fibroblasts from a GSD-II patient were incubated for 24 h with chimeric hGAA (SP-hGAA) in the medium (GAA activity 300 nmol/h/ml) with or without 5 mM mannose 6-phosphate. GAA activity and glycogen content were analyzed twice in duplicate. Untreated patient fibroblasts were used as control.

the secreted 110-kDa precursor hGAA is 50% lower than that of 76-kDa hGAA [20]. Although chimeric hGAA containing the human prealbumin signal peptide expressed hGAA in cells and the medium (Fig. 2A, lanes 11 and 12), we detected no GAA activity (not shown) and did not analyze it further. Western blot analysis of cellular and secreted hGAA in 293 cells transfected with the chimeric hGAA expression vectors demonstrated normal migration, consistent with unaltered glycosylation and processing of chimeric hGAA (Fig. 2A). These data were consistent with normal modification of chimeric hGAA, despite the increased rate of secretion and presumably shortened residence in the Golgi [5]. Normal modification by glycosylation and mannosylation of hGAA is critical to the mannose 6-phosphate receptor-mediated uptake of hGAA by cardiac and skeletal muscle in GSD-II [5,20].

High-Level, Persistent Secretion of Chimeric hGAA in Immunocompetent GAA-KO Mice

We evaluated the secretion of chimeric hGAA containing the hAAT signal peptide (SP-hGAA) *in vivo* with an AAV2/8 vector containing a universally active promoter (AAV-CBhAAThGAApA; Fig. 1A). We administered AAV-CBhAAThGAApA or AAV-CBhGAApA (encoding native hGAA) intravenously (1×10^{11} vector particles) to 3-month-old GAA-KO/SCID mice. We used GAA-KO/SCID mice initially to avoid any impact of neutralizing antibodies against hGAA [21]. GAA activity was increased with the vector encoding SP-hGAA in the plasma of GAA-KO/SCID mice (not shown). Western blot analysis of plasma detected higher levels for the AAV vector encoding SP-hGAA (Fig. 2B); however, lower levels of hGAA were present in liver with that vector (Fig. 2C). Thus, Western blot analysis confirmed the relative increase in secretion of chimeric hGAA from liver into plasma, in comparison to native hGAA.

Subsequently we administered an AAV vector containing a liver-specific promoter to drive liver-restricted SP-hGAA expression (AAV-SPhGAApA) intravenously to immunocompetent GAA-KO mice. This liver-specific promoter had previously expressed sustained, high-level hGAA in immunocompetent GAA-KO mice by virtue of avoiding immune responses against hGAA in mice [16]. Secreted hGAA was sustained in the plasma of GAA-KO mice for 24 weeks at >20 μg/ml (Fig. 2D). As few as 3×10^{10} particles of AAV-SPhGAApA produced secreted hGAA as detected by Western blotting of plasma (not shown). The presence of hGAA in multiple tissues for at least 24 weeks following vector administration was consistent with the persistent secretion and uptake of hGAA with AAV-SPhGAApA (Fig. 2E).

AAV vectors containing the liver-specific promoter used in AAV-SPhGAApA prevented an antibody response against coagulation factor IX (FIX) in hemophilia B mice and dogs [22,23]. This liver-specific promoter contains a thyroid hormone-binding globulin promoter sequence, two copies of an α1-microglobulin/bikunin enhancer sequence, and a leader sequence [24]. Several factors determine the ability to avoid antibody responses against foreign protein by liver-specific expression. Higher levels of FIX were associated with the induction of tolerance to liver-specific FIX expression [25]. Acquisition of tolerance to FIX required induction of regulatory $CD4^+$ T cells, most likely $CD25^+/CD4^+$ T regulatory cells, which suppressed neutralizing antibody formation [25]. The sinusoidal endothelial cells in liver are hypothesized to initiate tolerance through activation of naive T cells that do not differentiate into effector cells, because the liver is unique in its role in the induction of tolerance [26].

An AAV2/8 pseudotyped vector produced markedly higher hGAA levels in comparison to an AAV2/2 vector [14]; hence, an AAV2/2 vector could demonstrate the lower limits of efficacy for chimeric hGAA. In a direct comparison of AAV2/2 vectors administered intravenously (1×10^{11} particles), AAV-SPhGAApA produced chimeric hGAA at levels detectable in plasma (Fig. 2F); however, AAV-LSPhGAApA [16], containing native hGAA, did not secrete detectable hGAA (not shown). An increased antibody response was provoked by native hGAA expression in comparison to SP-hGAA expression (0.33 ± 0.13 vs 0.08 ± 0.06). Chimeric hGAA provoked a slightly positive ELISA (0.08 ± 0.06 vs 0.02 ± 0.02 for PBS); however, only two of five mice with a positive ELISA response cleared hGAA from the plasma following AAV-SPhGAApA administration (not shown). The AAV2/2 vector encoding chimeric hGAA demonstrated the possibility of avoiding an anti-hGAA antibody response without using novel AAV serotypes.

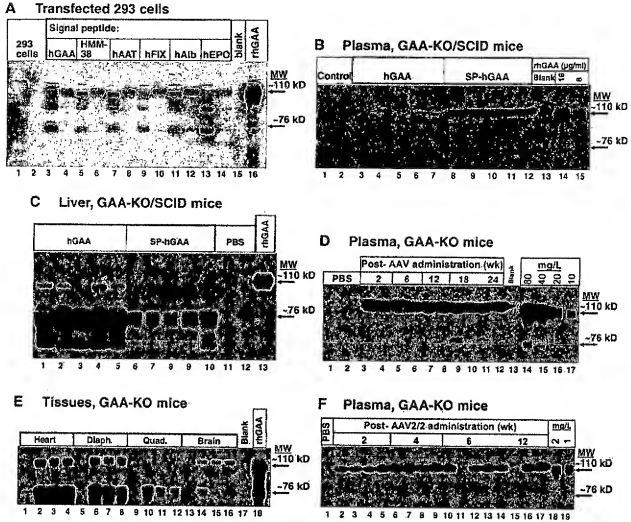

FIG. 2. Western blot analysis of chimeric hGAA. (A) Cell lysate (odd lanes) and medium (even lanes) are shown for 293 cells transfected with AAV vector plasmids encoding chimeric hGAA. The standard was 125 ng of recombinant hGAA (rhGAA). (B) Plasma of GAA-KO/SCID mice 1 week following administration of 1×10^{11} particles of either AAV-CBhGAApA (hGAA, $n=5$) or AAV-CBSPhGAApA (SP-hGAA, $n=5$). Each lane represents one mouse, and the controls were PBS-treated GAA-KO mice ($n=2$). (C) Liver from GAA-KO/SCID mice shown in (B), 7 weeks following vector administration. The standard was 125 ng of recombinant hGAA (rhGAA). (D) Plasma of immunocompetent GAA-KO mice from 2 to 24 weeks following administration of 5×10^{11} particles AAV-SPhGAApA ($n=2$). Control mice were PBS-injected, age-matched GAA-KO mice ($n=2$). Each lane represents an individual mouse. (E) Tissues analyzed 24 weeks following administration of AAV-SPhGAApA (5×10^{11} vector particles; $n=3$). The control was a PBS-injected, age-matched GAA-KO mouse ($n=1$). The standard was 125 ng of recombinant hGAA (rhGAA). (F) Plasma of immunocompetent GAA-KO mice from 2 to 12 weeks following administration of AAV-SPhGAApA pseudotyped as AAV2/2 (1×10^{11} particles; four of five transduced mice are shown). The control was a PBS-injected, age-matched GAA-KO mouse ($n=1$).

Long-Term Efficacy With Fewer Particles of an AAV Vector Encoding Liver-Restricted, Chimeric hGAA

The efficacy of gene therapy in GSD-II has been associated with elevated hGAA activity in plasma [27]. As few as 3×10^{10} particles of AAV-SPhGAApA elevated plasma GAA activity for 12 weeks following vector administration, in comparison to the level for mock-treated GAA-KO mice (Fig. 3; $P=0.02$ at 2 weeks, $P=0.02$ at 6 weeks, and $P=0.04$ at 12 weeks; Wilcoxon rank sum). Anti-GAA antibodies were absent (not shown), indicating the absence of a complicating humoral immune response to introduced hGAA.

Consistent with the hypothesis that increased plasma levels of the lysosomal enzyme would enhance efficacy, introduction of chimeric hGAA with an AAV vector improved the delivery of hGAA to skeletal muscle. We modified GAA by substituting a signal peptide from hAAT for its own, which improved the efficacy of an AAV vector

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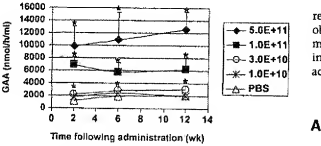

FIG. 3. Secretion of hGAA in GAA-KO mice with an AAV2/8 vector encoding chimeric hGAA. Mean ± SD is shown. Control mice were age-matched, PBS-injected GAA-KO mice. Plasma GAA was assayed for GAA-KO mice following administration of AAV-SPhGAApA [5.0×10^{11} ($n=6$), 1.0×10^{11} ($n=7$), 3.0×10^{10} ($n=4$), or 1.0×10^{10} ($n=4$) particles]. *Significant difference for the level in a group of vector-injected GAA-KO versus PBS-injected GAA-KO mice.

following systemic administration. The enhancement of secretion was similar to that seen from linking the fibronectin signal peptide to galanin, because intraparenchymal administration of an AAV vector encoding that modified galanin corrected a seizure phenotype in mice [28].

Long-term correction of glycogen storage in GSD-II reflects efficacious replacement of GAA, and therefore reductions in glycogen content were correlated with introduced GAA activity. GAA activity was markedly elevated in the heart and diaphragm 24 weeks following AAV-SPhGAApA administration, whereas GAA activity was approximately equivalent to the level for normal mice [13,14] in quadriceps (Fig. 4A). GAA activity was significantly elevated in all tissues analyzed by the administration of 1×10^{11} vector particles ($P<0.01$ for liver, heart, diaphragm, and quadriceps and $P=0.01$ for brain; Wilcoxon rank sum). Glycogen content was significantly reduced to normal levels in the heart and to near-normal levels in the diaphragm following administration of only 1×10^{11} vector particles, in comparison to the glycogen content for mock-treated GAA-KO mice for each tissue (Fig. 4B, $P<0.01$; Wilcoxon rank sum). The levels of hGAA achieved with the AAV2/2 vector encoding chimeric hGAA did not reduce glycogen content in striated muscle (data not shown). Quadriceps was more resistant to correction than the diaphragm (Fig. 4B), as observed following adenovirus vector or AAV2/8 vector-mediated gene therapy in GSD-II mice [14,16,27]. Intriguingly, the brain exhibited significantly increased GAA activity (Fig. 4A, $P=0.01$; Wilcoxon rank sum), and

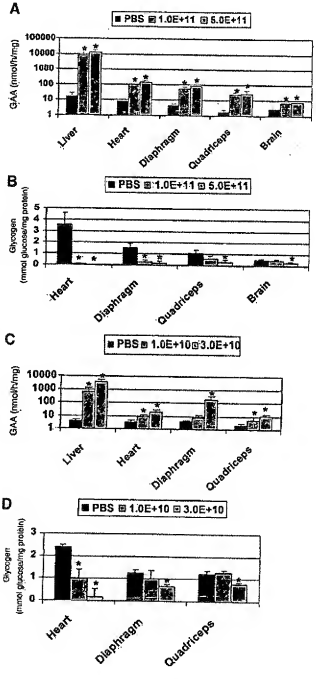

FIG. 4. Biochemical correction with chimeric hGAA in GAA-KO mice. Mean ± SD is shown. Control mice were age-matched, PBS-injected GAA-KO mice. *Significant difference for the level in a group of vector-injected GAA-KO versus PBS-injected GAA-KO mice. (A) Tissue GAA was assayed for GAA-KO mice 24 weeks following administration of AAV-SPhGAApA [5.0×10^{11} particles ($n=6$) or 1.0×10^{11} particles ($n=7$)], in comparison to mock-treated GAA-KO mice (PBS, $n=4$). (B) Glycogen content in tissues for mice in (A). (C) Tissue GAA activity in GAA-KO mice 18 weeks following administration of AAV-SPhGAApA [3.0×10^{10} particles ($n=4$) or 1.0×10^{10} particles ($n=4$)], in comparison to mock-treated GAA-KO mice (PBS, $n=4$). (D) Tissue glycogen quantitation for mice in (C).

glycogen content was reduced in the brain following administration of 5×10^{11} vector particles, in comparison to the respective levels for mock-treated GAA-KO mice in brain (Fig. 4B, $P=0.01$; Wilcoxon rank sum).

Uptake of secreted hGAA can be demonstrated only if plasma levels achieve a therapeutic threshold. Therefore, we administered lower numbers of AAV-SPhGAApA vector particles to GAA-KO mice in a dose–response experiment. The number of vector particles required to elevate GAA activity significantly in the liver and striated muscle was 3×10^{10}, in comparison to mock-treated GAA-KO mice (Fig. 4C, $P=0.02$; Wilcoxon rank sum). The urinary biomarker for GSD-II, Glc_4, was reduced following administration of 3×10^{10} vector particles (0.22 ± 0.11 mmol/mol creatinine versus 0.42 ± 0.17 for mock-treated GAA-KO mice). The glycogen content of the heart was reduced by administration of only 1×10^{10} vector particles, in comparison to mock-treated GAA-KO mice (Fig. 4D, $P=0.02$; Wilcoxon rank sum). The threshold to reduce heart ($P<0.02$), diaphragm ($P=0.02$), and quadriceps ($P=0.02$) glycogen content significantly, in comparison to levels for mock-treated GAA-KO mice, was 3×10^{10} particles (Fig. 4D; Wilcoxon rank sum). The analogous vector encoding native hGAA failed to reduce the glycogen content in quadriceps, when we administered an equivalent number of vector particles (data not shown). Thus, the substitution of chimeric hGAA for native hGAA increased the efficacy of the AAV2/8 vector.

The feasibility of gene therapy in GSD-II would be enhanced by reducing the requirement for vector needed to achieve efficacy. Pompe disease/GSD-II features a very high threshold for the correction of the underlying enzyme deficiency, indicating that reducing the level of treatment needed to achieve efficacy is critical [29,30]. The main benefit of delivering chimeric hGAA was to reduce the number of vector particles required to achieve efficacy by at least threefold. We previously demonstrated efficacy with a higher number of particles of AAV-LSPhGAApA (1×10^{11} vector particles), which encodes native hGAA [16]; whereas the number of vector particles currently required to achieve efficacy with AAV-SPhGAA was reduced to approximately 3×10^{10}.

Recently characterized AAV serotypes offer improved tissue tropism, which reduced the therapeutic threshold in terms of vector particles. Traditional vectors utilizing AAV2 terminal repeat (TR) sequences can be efficiently cross-packaged as other serotypes [6,31,32]. If the AAV2 vector was cross-packaged as AAV1 (AAV2/1), AAV2/6, AAV2/7, or AAV2/8, it achieved increased transduction of muscle fibers compared to traditional AAV2/2 vectors [31–34]. AAV2/1, AAV2/6, AAV2/7, and AAV2/8 transduced liver with improved efficiency, compared to AAV2/2, following intravenous injection [31–34]. AAV2/8 vectors delivered genes to the liver approximately 100-fold more efficiently than traditional AAV2/

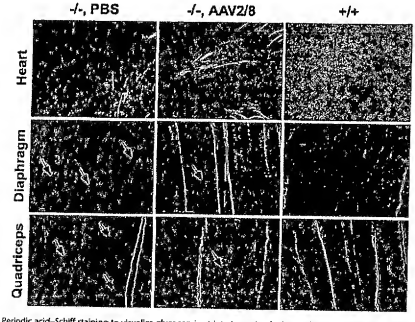

FIG. 5. Glycogen staining. Periodic acid–Schiff staining to visualize glycogen in striated muscle of mice analyzed at 9 months of age. GAA-KO mice were injected with AAV-SPhGAApA packaged as AAV2/8 (5×10^{11} particles) or PBS (for mock-treated controls). Two sections were examined for each of three mice from each group, and a typical result is presented. Original magnification: 100× (heart) or 200× (diaphragm and quadriceps). Arrows indicate foci of glycogen vacuolation.

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2 vectors in knockout mice, including GAA-KO/Pompe disease mice [14,32]. Indeed, the vast majority of hepatocytes could be transduced by a single administration of an AAV2/8 vector in adult mice [32,35]. A single report questions this claim, because an AAV2/8 vector containing a universal promoter to drive G6Pase expression did not promote long-term survival in G6Pase-KO mice following neonatal administration; moreover, when the same vector was cross-packaged as AAV2/1 it did sustain G6Pase-KO mice long-term [36]. However, an AAV2/8 vector did prolong survival in G6Pase-KO mice when delivered at 2 weeks of age [37]. Direct comparison of AAV2/7, AAV2/8, and AAV2/9 vectors in ornithine transcarbamylase deficiency mice concluded that AAV2/8 transduced the liver at least as efficiently as the other two serotypes [38]. Therefore, it is widely accepted that AAV2/8 achieves the highest level of transduction in the liver of adult mice, although AAV2/1 might transduce hepatocytes more efficiently in neonatal mouse liver.

Correction of Histomorphology Accompanied hGAA Expression With AAV-SPhGAApA

Glycogen staining revealed a reduction in glycogen vacuolation in striated muscle following AAV-SPhGAApA administration (−/−, AAV2/8), including heart, diaphragm, and quadriceps, in comparison to sham-treated (−/−, PBS) GAA-KO mice (Fig. 5). Although vector DNA was detected at >30 vector genomes/cell in liver and up to 1 vector genome/cell in striated muscle (Figs. 6A and 6B), vector RNA was barely detectable in skeletal muscle (Fig. 6C). The higher liver GAA activity, in comparison to quadriceps, was associated with high-level hGAA expression driven by the LSP in liver (Fig. 4A); however, leaky transgene expression contributed at least in part to hGAA activity in skeletal muscle. The hGAA RNA in the heart likely contributed to GAA activity, as did receptor-mediated hGAA uptake, which is very efficient in heart [27,30]. High-level hGAA expression with a second-generation adenovirus vector containing this LSP reduced glycogen content in the heart and skeletal muscle of GAA-KO mice, consistent with very high level hGAA expression in liver and cross-correction of skeletal muscle through receptor-mediated uptake [27].

Significantly reduced glycogen content in the brain, in comparison to mock-treated GAA-KO mice (Fig. 4B), was associated with the presence of vector DNA and vector RNA in the brain (Figs. 6B and 6C). It is generally accepted that lysosomal enzymes such as GAA are unlikely to cross the blood–brain barrier [39]. The correction of GAA deficiency and reduction of glycogen content in the brain indicated that the AAV2/8 vector transduced either the brain or closely associated tissues such as vascular endothelium following intravenous administration. AAV2/8 vectors can penetrate endothelial barriers and crossed the blood–brain barrier to transduce neurons, albeit with low efficiency [35,40]. If an AAV2/8 vector penetrated the central nervous system and expressed therapeutically relevant GAA, it could potentially ameliorate the neuromuscular involvement in GSD-II [1].

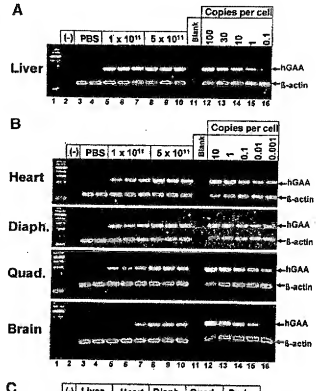

FIG. 6. Vector biodistribution and pattern of hGAA expression. GAA-KO mice were analyzed 24 weeks following administration of 1×10^{11} or 5×10^{11} vector particles. Control mice were age-matched, PBS-injected GAA-KO mice. A negative control PCR (−) received no input DNA (lane 2). The molecular weight marker is a 100 bp ladder (lane 1). The fragment sizes for hGAA and for mouse β-actin are indicated (arrows). (A) Vector DNA semiquantitation in the liver. (B) Vector DNA semiquantitation in other tissues. (C) RT-PCR detection of vector RNA in the indicated tissues. The first sample for each tissue is from a control mouse.

Currently, the substitution of a human α1-antitrypsin signal peptide for the hGAA signal peptide accomplished secretion of hGAA with lower numbers of vector particles. Furthermore, as demonstrated for an AAV2/2 vector here, chimeric hGAA induced tolerance more effectively than unmodified hGAA in immunocompetent GAA-KO mice. Pompe disease/GSD-II presents an opportunity for a clinical trial of gene therapy in a prototypical lysosomal storage disorder,

because efficacy and persistence have been achieved in available animal disease models [11,14–16,41,42]. The previously recognized hurdles of efficacious GAA production [11,14], tolerance to introduced GAA [15,16], and persistence of GAA expression in immunocompetent GAA-KO mice [15,16,43,44] have been cleared in the current study.

Methods

Construction of hGAA vectors with the different signal peptide. Two fragments were ligated to produce each novel signal peptide: a bridging sequence from the human GAA cDNA (fragment I) and the unique signal peptide sequence (fragment II). Fragment I was synthesized as a 287-bp hGAA sequence beginning at the 3′ terminus of the hGAA signal peptide sequence and extending to a unique *Sac*II site. Fragment I was amplified by PCR from a vector plasmid containing the human GAA cDNA (pAAV-CBhGAApA [6]), with the following primers: forward, 5′-GCTGCAA-*AGCTT*gggcacatcctactccac-3′, which has an introduced *Hin*dIII site at its 5′ end, and reverse, (5′-ccttgatccccgcttttgcagggatgtage-3′. the resultant per fragment was digested with *Hin*dIII and *Sac*II and gel-purified to produce fragment i.

Fragment II was unique to each plasmid construct. For the pAAV-CBhAATHGAApA vector plasmid fragment II was synthesized with the following oligonucleotides: forward oligonucleotide—containing the human α1-antitrypsin signal peptide sequence [human α1-antitrypsin cDNA: position 39–110 (72 bp), GenBank X01683], 5′-CT*GAGG-TACCTGCCACC*-**ATGCCGTCTTCTGTCTCGTGGGGCATCCT-CCTGCTGGCAGGCTGTGCTGCCTGGTCCCTGTCTCCCTGGCT-***AAGCTT*CGAT-3′, and reverse, 5′-*ATCGAAGCTT*AGCCAGGGAG-3′. The forward oligonucleotide has a 5′ *Kpn*I site, followed by an optimal Kozak sequence (GCCACC), the human α1-antitrypsin leader sequence, and a 3′ *Hin*dIII site. The forward and reverse oligonucleotides were incubated with Kienow DNA polymerase in the presence of dNTPs at 37°C for 15 min to complete second-strand synthesis and subsequently digested with *Kpn*I and *Hin*dIII. Fragments II incorporating the HMM38, hEPO, hAlb, and hFIX signal peptides were designed using the above-mentioned strategy. The forward oligonucleotides for producing fragment II for each of those vector plasmids contained the following sequences in place of the boldface nucleotides shown for forward oligonucleotide of pAAV-CBhAATHGAApA (above):

(1) pAAV-CBSP38hGAApA (HMMSP38 leader sequence [17]: ATGTGGTGGCGCCTGTGGTGGCTGCTGCTGCTGCTGCT-GCTGTGGCCCATGGTGTGGGCC (63 bp).
(2) pAAV-CBhEPO (hEPO leader sequence, position 182–262): GenBank NM000799 (81 bp).
(3) pAAV-CBSPhFIXhGAApA (human coagulation factor IX leader sequence, position 1–138): GenBank M11309 (138 bp).
(4) pAAV-CBSPalbuminhGAApA (human prealbumin cDNA, position 40–93): GenBank NM000477 (54 bp).

The purified ligation products from fragment I and fragment II were ligated with the *Kpn*I–*Sac*II fragment from the pAAV-CBhGAApA vector plasmid. The ligation was transformed into STBL2 competent cells (Invitrogen, Carlsbad, CA, USA) to avoid deleting the AAV TR sequences, and plasmid DNA was purified.

The ~816-bp LSP contains a thyroid hormone-binding globulin promoter sequence and two copies of an α1-microglobulin/bikunin enhancer sequence [24]. The LSP was subcloned from pAAV-LSP-cFIX [kind gift from Dr. Inder Verma (Salk Institute, La Jolla, CA, USA)] to generate pAAV-LSPhGAApA [16]. The pAAV-SPhGAApA vector plasmid was constructed by ligation of the ~0.8-kb *Kpn*I to *Xba*I fragment containing the LSP from pAAV-LSPhGAApA with the ~4.7-kb *Kpn*I to *Xba*I fragment from the pAAV-CBhAATHGAApA vector plasmid.

Preparation of AAV vectors and analysis in cultured patient cells. Briefly, 293 cells were transfected with an AAV vector plasmid, the AAV packaging plasmid pSE18-VD 2/8 [32] (courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA), and pAdHelper (Stratigene, La Jolla, CA, USA). Cell lysate was harvested 48 h following infection and freeze-thawed three times and isolated by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against three changes of Hanks' buffer, and aliquots were stored at −80°C. The number of vector DNA-containing particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the NIH.

The receptor-mediated uptake of chimeric hGAA was analyzed in Pompe disease patient cells as described [20]. Fibroblasts from a GSD-II patient were grown in DMEM containing 10% FBS and incubated for 40 h with the medium of transfected 293 cells producing chimeric hGAA with activity of 300 nmol/h/ml, either with or without the addition of mannose 6-phosphate (5 mM) to inhibit mannose-6-phosphate receptor-mediated uptake. GAA activity and glycogen in cultured patient fibroblasts were analyzed as described [6].

Analysis of AAV vector stocks In vivo. The AAV vector stocks were administered intravenously (via the retroorbital sinus) to 12-week-old GAA-KO mice [44]. At the indicated time points postinjection, plasma or tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

GAA activity and glycogen content were analyzed as described [11].

Western blotting analysis of hGAA in plasma and tissues and semi-quantitation of AAV vector DNA by PCR and RT-PCR were performed as described [18].

The ELISA was performed as described [6]. All samples yielded absorbance values that were within the linear range of the assay at this dilution. GIc_4 analysis was performed as described [14].

Statistical analyses. The data were analyzed using both parametric (ANOVA) and nonparametric (Wilcoxon rank sum) testing. Parametric and nonparametric methods were used because of the relatively small number of mice studied (total sample size $n = x$). The results were similar using both methods; specifically, P values that were <0.05 using ANOVA were also <0.05 using nonparametric methods. The nonparametric testing provided more conservative (higher) P values and therefore those results are presented. All P values are two-tailed. The analysis was completed using STATA 8.2 (College Station, TX, USA).

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